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Research Article

A Novel Chemical Entity, that Reverses Warburg Metabolism by Disrupting VDAC1/HK2 Interaction through "Toposteric Effect" in Cancer

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ABSTRACT

Background: Acidic pH and low oxygen levels due to the Warburg effect have been shown to impart resistance to certain anti-cancer therapies for solid tumors, such as radiation and a variety of chemotherapeutic drugs. Cancer immunotherapy represents one of the most exciting advancements in recent cancer therapy. However, despite these achievements, the numbers of patients with effective cure are very low for patients with solid tumors. Recent studies pointed out that the main cause for this low efficiency is the Tumor Microenvironment (TME), with metabolic changes, and immune evasion that renders solid tumor eradication a real challenge. Drugs targeting the inhibitory TME are urgently needed in combination with immunotherapy, as well as other conventional therapies like chemotherapy and cancer targeted growth blockers.

Methods: In this study we used an inhibitor of Hexokinase-2 binding to the mitochondrial VDAC1 channel (VDA-1275), that is shown to block cancer cell proliferation, induce apoptosis in cancer cells, and change TME from a protumor to a pro-immunological environment. Our studies demonstrate a significant tumor growth inhibition and survival prolongation, combined with a strong safety profile *in-vivo*. In addition, a synergistic anti-cancer effect was demonstrated, using a 3D cell culture with human hepatic cancer cell organoids, when VDA-1275 was combined with low levels of Cisplatin or Sorafenib, as examples for chemotherapy and targeted therapy treatments, respectively. In addition, synergistic effect was demonstrated, using a colon cancer syngeneic mice model treated with combination of VDA-1275 and Cisplatin *in-vivo*.

Results: Our results suggest that VDA-1275 is a novel compound that effect cancer cells directly and indirectly by changing the TME to a pro-immunogenic environment.

Conclusion: VDA-1275 may be used as a standalone drug, or in combination therapy that will allow more effective and safe treatment of patients with solid tumors. We coined the word and concept of "Toposteric" effect as the use of small molecules or peptides which interact with a receptor or ligand binding site, avoiding the possibility of a pro-pathological harmful anchoring without affecting its active site.

Keywords: Warburg effect; Tumor microenvironment; Hexokinase-2; VDAC1; Hexokinase-1

Abbreviations

BMDM: Bone Marrow-derived Macrophges; CI: Combination Index; HK1/2: Hexokinase ½; IC50: Inhibition Concentration of 50%; IP: Intraperitoneal; NCE: New Chemical Entity; PO: Per os; TAMs: Tumor-Associated Macrophages; TGI: Tumor Growth Inhibition; TK: Toxico Kinetics; TME: Tumor Microenvironment

INTRODUCTION

Due to the aerobic glycolysis in cancerous cells, the extracellular pH and oxygen concentration decreases as we move away from the vasculature into the solid tumor space. Acidic pH and low oxygen levels have been shown to impart resistance to certain anti-cancer therapies such as radiation and a variety of chemotherapeutic drugs [1]. Moreover, a tumor comprises stromal cells that contain

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cancer-associated fibroblasts, as well as endothelial cells, pericytes and immune inflammatory cells, which together comprise about 50% of the cell population in tumor tissues. These stromal cells are indispensable for tumor growth and metastasis; moreover, they promote immune tolerance and chemoresistance in tumors [2]. This acquired resistance to treatment applies also to immune therapies. Clinical trials have shown that cancer immunotherapy can induce unprecedented durable responses in patients with a variety of cancers. However, objective responses were only observed in a small proportion of patients. In many cases, patients fail to establish a sustained anti-tumor immunity and to achieve a long-lasting clinical benefit. Metabolic features of cancer cells, and the tumor microenvironment impose constraints on immune cell metabolism that can favor immunosuppressive phenotypes and block antitumor responses [3-5]. Most tumors are refractory to adoptive cellular therapy with immune cells and the molecular mechanisms underlying resistance was identified as tumor glycolysis, which is the pathway associated with immune resistance in melanoma and lung cancer patient samples, poorly infiltrated by T cells [5,6].

This resistance necessitates novel agents and combinations to address the unmet clinical challenge. The immediate surrounding of the tumor-the Tumor Microenvironment (TME)-has characteristics that are different from the rest of the body. Agents secreted from the tumor (e.g., metabolites, cytokines) interact with immune cells in its vicinity making it resistant to intervention by the host immune system. Given the inherent complex TME, heterogeneous nature and stemness of tumors, neoantigen recognition alone cannot eliminate solid tumors *in vivo*. Primary and acquired resistance necessitates novel agents and combinations to address this unmet clinical challenge. There is a swapping recognition that a new classes of drugs, that will target the immune-inhibitory TME are greatly needed. Such drugs will enable an anti-cancer immune response in tumors that are currently non-responsive to chemotherapy, targeted therapy, or immunotherapy. These new drugs, in addition to their direct effects, will be especially effective in combination with the already approved 1st line cancer treatments and such combinations, once approved, could offer hope for millions of cancer patients worldwide for whom no effective treatment exists today.

Normal cells oxidize pyruvate into adenosine triphosphate and carbon dioxide in the presence of oxygen in mitochondria. However, cancer cells increase glucose uptake and preferentially metabolize pyruvate to lactate even in the presence of oxygen, a process named the Warburg effect, to maintain a slightly acidic micro-environment, and to use the excess glucose as a carbon source for anabolic processes, both needed to support cell proliferation and metastasis [7]. Hexokinases are the first glycolysis rate-limiting enzymes that irreversibly phosphorylate glucose to glucose-6-phosphate. Four distinct HK isoforms have been discovered in mammals. They are scattered throughout the cell in various locations. In normal cells Hexokinase 2 (HK2) is found as traces mainly in the cytosol where it controls glycogen synthesis [8]. Upon transformation to cancerous cells, HK2 molecules undergo overexpression and are particularly situated on the outer mitochondrial membrane by interacting with the mitochondrial Voltage-Dependent Anion Channel 1 (VDAC1), which has the advantage of avoiding product inhibition and improving apparent binding with ATP. This interaction also suppresses the release of the proapoptotic molecule cytochrome C from the mitochondria to the cytosol, hence increasing aerobic glycolysis in tumor cells and reducing apoptosis [8-10]. HK2 is expressed at a high level in many different cancer types, including Liver, Lung, Breast, pancreatic cancer, ovarian and others [11-13]. Moreover, HK2 is required for tumor initiation and maintenance and its systemic deletion is therapeutic in mouse models of cancer [14-15]. Blocking the interaction between HK2 and VDAC1 reduces proliferation and induces apoptosis while also affecting immune cells within the TME, supporting a pro-immunogenic environment.

We have previously described the development of a selective small molecule (VDA-1102) that blocks the interaction between HK2 and VDAC1 [16]. This compound has a short half-life in blood and is therefore suitable for topical use and has been developed as a topical ointment through a successful Phase 2B clinical study for the indication of advanced Actinic Keratosis. The study (NCT 03538951) demonstrated a 40% Complete Clearance and 80% lesions reduction in 50% of the population with an attractive safety profile. Here we describe a "toposteric" compound VDA-1275, which is a potent soluble new chemical entity, that selectively modulates VDAC1/HK2 interaction with a significant higher affinity for the HK2 binding site. It is highly potent in vitro against a broad range of tumor cell types and demonstrates significant selectivity for VDAC1/HK2 system over VDAC1/HK1 system. By the term "toposteric" we mean that VDA-1275 prevents the harmful interaction of HK2 with the mitochondrial VDAC1 molecule that initiates the Warburg effect and leads to cancer formation. Instead, VDA-1275 treatment releases HK2 back to its original cytosolic location. VDA-1275 is chemically unrelated to VDA-1102 that is used for topical treatment, and presents a different set of pharmacokinetic characteristics and is being developed as a systemic drug for treatment of solid tumors.

METHODS

Cellular cleaved caspase 3/7 activity

The effect of VDA-1275 on apoptosis induction was tested by measuring the increase in cellular levels of cleaved caspase-3/7 activity within 6 hours of treatment, using Caspase Glo (Promega). Cells were treated with VDA-1275 at 0.03 and 0.1 μM, medium was used as untreated control.

Glycolysis reduction test

Analysis of Extra-Cellular Acidification Rate (ECAR) was performed using Seahorse XF analyzer to assess the effects of VDA-1275 on glycolysis in the B16 mouse melanoma cancer cell line. Cells were treated with serial addition of glucose, oligomycin, and 2-DG to measure basal glycolysis, maximal glycolytic capacity, and nonglycolytic ECAR.

Cancer cell proliferation

Two human cell lines, the Non-Small Cell Lung Cancer (NSCLC) H358, and the prostate cancer PC-3 cell lines, as well as two murine cancer cell lines: the colorectal CT26, and the colon adenocarcinoma cells MC38, were used. Cells were incubated with a five-fold range of VDA-1275 concentrations for 72 hours and XTT cell viability assay was used to determine the IC50 for each cell line compared to untreated control.

Macrophages phenotype *ex-vivo*

Bone Marrow Derived Macrophage (BMDM) cells were grown as

described previously [17], using 50 ng/ml recombinant macrophage Colony Stimulating Factor (M-CSF). Cells were plated and primed with LPS (50 ng/ml) for 3hr-4 hr, followed by treatment with Peptidoglycan (40 µg/ml) for 6 hr. Cells were treated with elevated amount of VDA-1275 as described or medium as control. Cell supernatants were analyzed by ELISA for IL-1β (BioLegend) and IL-18 (MBL International) levels. To study the effect of VDA-1275 on macrophages that were prone to become M2 macrophages, BMDM were treated with the Th2- inducing cytokine IL-4. Cells were then treated with elevated levels of VDA-1275 for 24 hrs and the effect on the expression of the M2-phenotype genes Fizz1, Chi313 and Cdh-1 was studied using Real time PCR. Results were normalized to actin.

Percentage of central memory CD8 T-cells *ex-vivo*

Splenocytes were isolated from a transgenic mouse strain that carries a T-cell receptor transgene specific for the mouse homologue pmel-17. Mice splenocytes were primed with their specific peptide for 24 hours in the absence or presence of VDA-1275 (0.1µM). Cells were analyzed on FACS for their activation state using CD44 staining and for the Central Memory biomarker CD62L.

A syngeneic MC38 mouse model

C57BL/6 immuno-competent mice were inoculated subcutaneously at the right flank with MC38 cells ($1x10^6$) for tumor development. Five days after tumor inoculation, 20 mice with tumor size ranging from 30-61 mm^3 (average tumor size 46 mm^3) were selected and assigned into 2 groups using stratified randomization (N=10), based upon their tumor volumes. Treatments started from the day of randomization (defined as D0) and included the vehicle group (25%PEG400+75% Labrafac Lipophile WL 1349) PO, QD x 25 days and the VDA-1275 300mpk PO, QD x 25 days. The tumor sizes were measured three times per week during the treatment period. Survival was monitored with tumor volume exceeding 2000 mm^3 as endpoint.

Animal Source: Mice were purchased from Shanghai Lingchang Biotechnology Co. Ltd. The animal study was performed by ChemExplorer Company, Shanghai ChemPartner Co., with their place of business at 998 Halei Road, Building Number 5, Shanghai 201203, China. Ethical approvals: The study was performed following to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of ChemPartner in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). After tumor inoculation, the animals were checked daily for morbidity and mortality. At the time of routine monitoring, the animals were checked for any effects of tumor growth and treatment on normal behavior such as mobility, body weight gain/loss (body weights was measured twice weekly or every other day), eye/hair matting and any other abnormal effect. Death and observed clinical signs were recorded. Any serious adverse reactions or death were reported to Vidac immediately."

Termination: Animals showing obvious signs of severe distress and/or pain were humanely sacrificed by using CO_2 at a fill rate of 30%-70% displacement of the chamber volume per minute, added to the existing air in the chamber. In case of following situation, the animals were euthanized following discussion with Vidac: 1. Animals have lost significant body mass (emaciated), Obvious body weight loss>20%. 2. Animals cannot get adequate food or water. 3. Tumor volume>2000 mm^3.

A syngeneic CT-26 mouse model

BALB/c immuno-competent mice, 8-10 weeks old females were inoculated subcutaneously at the right flank with CT-26 cells ($1x10^6$) for tumor development. When average tumor size reach 50 mm^3, mice were assigned into 6 groups (N=10) using stratified randomization, and daily treatment start. Mice were treated with Cisplatin twice a week by intraperitoneal route of administration (IP), and with VDA-1275 daily Per os (PO). Groups included the vehicle group (25%PEG400+75% Labrafac Lipophile WL 1349) PO, and saline IP (group 1), Cisplatin 0.5 mg/Kg IP (group 2), Cisplatin 0.5 mg/Kg and VDA-1275 400mpk PO (group 3), Cisplatin 1 mg/Kg (group 4), Cisplatin 1 mg/Kg and VDA-1275 400mpk (group 5) and VDA-1275 400mpk (group 6). Animals were tested for body weight, behavior and clinical signs every two days, as well as for food and water consumption. In addition, tumor size was measure every two days according to the formula: $V=AxB^2$, where A is the long dimension and B is the short one.

Ethical approvals: The study was performed following to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Termination: Animals showing obvious signs of severe distress and/or pain were humanely sacrificed by using CO_2 at a fill rate of 30%-70% displacement of the chamber volume per minute, added to the existing air in the chamber. In case of the following situation, the animals were euthanized following discussion with Vidac: 1. Obvious body weight loss>20%. 2. Animals cannot get adequate food or water. 3. Tumor volume>1800 mm^3.

A 3D organoid model in an extracellular matrix-like hydroscaffold (BIOMIMESYS®)

The human hepatocellular carcinoma cell line HepG2 was used in a matrix composed of hyaluronic acid and collagen I (BIOMIMESYS® Oncology, HCS Pharma cat. BIO_ONC_96_96_black). First, cells were seeded at 10,000 cells/well and incubated for 4 days at 37°C, 5% CO2, following treatment with a range of VDA-1275 concentrations during 72 hr, as indicated. Then, cells were tested for proliferation using Click-iT Plus EdU Alexa Fluor™ 488 Imaging Kit (Fisher Scientific, cat. 15234997), and viability was determined by staining nuclei with Hoechst 33342 at 10µg/mL (Invitrogen™ H3570, Fisher Scientific, cat. 11534886) and dead cells with DRAQ7 staining at 1.2µM (Ozyme, cat. BLE424001).

Next, two anti-cancer drugs were chosen to study their synergistic effect with VDA-1275: Sorafenib (Sigma Aldrich, cat. SML2653-5MG) and Cisplatin (TCI, cat. D3371-100M). The powders were solubilized in DMSO, at 200 times the final concentrations, to reach a final dilution of 0.5% DMSO. VDA-1275 was co-incubated with Sorafenib (1µM and 3µM), Cisplatin (5µM and 15µM), or cell media (0.5% DMSO) as vehicle control. For each treatment, increasing concentrations of VDA-1275 were added (3nM -1000 nM) or vehicle. Cells were incubated for 3 days followed by viability and proliferation tests, as described.

Results were obtained by confocal microscopy followed by image analysis. The images were acquired by the ImageXpress Micro Confocal system automated microscope (Molecular Devices), with 4 acquisition sites per well, using confocal 60µm pinhole imaging

method, objective 10. Three filters were used, DAPI (excitation 377/50nm, emission 447/60nm), FITC (excitation 475/34 nm, emission 536/40nm,), and Cy5 (excitation 631/28 nm, emission 692/40 nm). Images were post-treated with MetaXpress software (6.63.55 version). The maximal 2D projection was analyzed using multiwavelength cell scoring application module.

Synergistic effect was defined using the Response additivity method. This reference model is also known as Linear Interaction Effect and assumes that a positive interaction occurs when the drug combination elicits a greater effect than the sum of the individual drug's effects (EAB>EA+EB) [18].

RESULTS

VDA-1275 leads to cell apoptosis

The chemical structure and formula name of VDA-1275 is shown in Figure 1A. The effect of VDA-1275 on apoptosis induction was tested by measuring the increase in cellular levels of cleaved caspase-3/7 activity within 6 hours of treatment, showing over 50% and 300% elevation of activity using low VDA-1275 concentrations of 0.03 and 0.1 μM, respectively (Figure 1B). This early event is in line with a caspase-dependent apoptotic mechanism.

VDA-1275 inhibits cancer cell proliferation

The effect of VDA-1275 on cellular proliferation was tested using the XTT cell viability assay. Cells were incubated with VDA-1275 for 72 hours. Two human cell lines were tested, the Non-Small Cell Lung Cancer (NSCLC) H358 and the prostate cancer PC-3 cell lines, resulting in IC50 values of 0.009 and 0.053 μM, respectively. In addition, two murine Cancer cell lines, the colorectal CT26 and the colon adenocarcinoma cells MC38 cell lines, were also tested resulting in IC50 values of 0.049 and 0.133μM, respectively (Figure 1C).

VDA-1275 reduces glycolysis that leads to decreased proliferation of cancer cells

To test the effect of VDA-1275 on Glycolysis, the Extra-Cellular Acidification Rate (ECAR) was measured to study the lactic acid levels, formed during the conversion of glucose to lactate using the B16 mouse melanoma cancer cell line. Glucose was added to cells and cells were treated with 0.1% DMSO as vehicle control and with 1μM and 10μM VDA-1275. A clear dose response of VDA-1275 on glycolysis rate decrease as well as on the glycolytic capacity of the cells was observed (Figure 2).

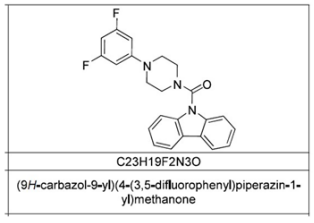
VDA-1275 EFFECTS ON IMMUNE CELLS

VDA-1275 induces M1 phenotype and inhibits M2 phenotype in macrophages

The effect of VDA-1275 on macrophage activity was studied. Metabolic changes of cancer cells, mainly the high levels of aerobic glycolysis, affect the tumor microenvironment and impose constraints on immune cell metabolism that can favor immunosuppressive phenotypes and block antitumor responses. However, it also inhibits the function of Tumor-Associated Macrophages (TAMs), that are ubiquitously present in solid tumors, thereby facilitating the immune evasion of malignant tumor cells. TAMs are generally divided into the pro-inflammatory M1 phenotype and the pro-tumor M2 phenotype. Large excess of lactate leads TAMs to adopt an immunosuppressive phenotype and collaborate with tumor cells to promote angiogenesis [19].

In order to study the direct effect of VDA-1275 on macrophages, we first used primed macrophages, in which activation of the NLRP3 inflammasome leads to secretion of the cytokines IL-1ß and IL-18. IL-1ß is a major pro-inflammatory cytokine, while IL-18 acts together with IL-12 to induce a Th1 immunological response that characterizes the M1 phenotype of macrophages. Bone Marrow-Derived Macrophage (BMDM) cells were grown using recombinant Macrophage Colony Stimulating Factor (M-CSF). Macrophages were treated with VDA-1275, followed by priming with LPS, and peptidoglycan. The effect on NLRP3 inflammasome was tested by measuring the levels of IL-1ß and IL-18. A significant elevation of IL-1ß and IL-18 was observed when VDA-1275 was added to the cells at concentrations of 0.03-1 μM and 0.1-0.3 μM, respectively (Figure 3A). An additional study with BMDM was performed to study the effect of VDA-1275 on macrophages that were prone to become M2 macrophages by treatment with the Th2 cytokine IL-4. Cells were treated with VDA-1275 for 24 hrs and the effect on the expression of the M2-phenotype genes Fizz1, Chi313 and Cdh-1 was studied using real-time PCR. Results demonstrated a strong and significant reduction in the expression of these three M2 type genes following VDA-1275 treatment, suggesting that VDA-1275 affects the metabolism of TAMs by shifting pro-tumor M2 macrophages to the pro-immunologic M1 type macrophages (Figure 3B).

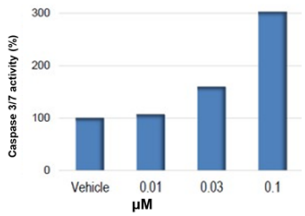
A



C23H19F2N3O

(9*H*-carbazol-9-yl)(4-(3,5-difluorophenyl)piperazin-1-yl)methanone

B



C

VDA-1275	human		murine	
Cell line	H358 Lung cancer	PC3 Prostate cancer	CT26 Colon Cancer (m)	MC38 Colon Cancer (m)
IC50 (μM)	0.009	0.053	0.049	0.133

Figure 1: VDA-1275 induces apoptosis in cancer cells. (A): The molecular structure and formula of VDA-1275; (B): Cellular activity of cleaved caspase-3/7 following treatment with VDA-1275; (C): XTT cell viability assay IC50s for VDA-1275 on various human and murine cell lines.

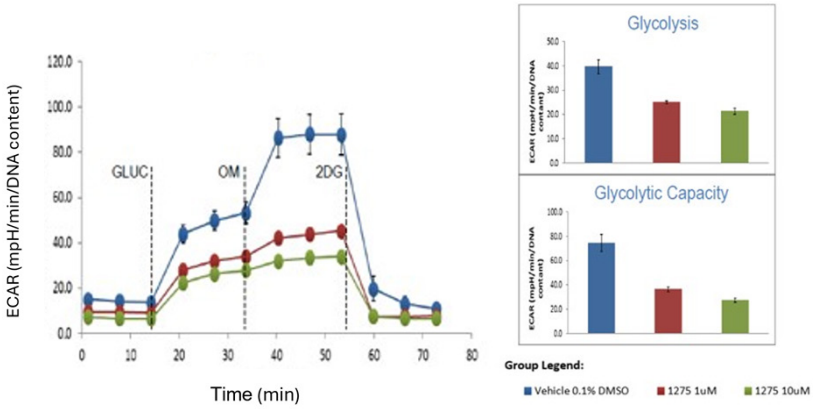


Figure 2: VDA-1275 reduces Glycolysis in cancer cells. Glycolysis levels measured via the Extra-Cellular Acidification Rate (ECAR) following treatment of the B16 mouse melanoma cancer cells with VDA-1275. Sequentially, glucose (GLUC) is added to start glycolysis, Oligomycin (OM) is added for maximum glycolytic capacity, and 2DG is added to block glycolysis. The inserts show the effect of VDA-1275 on glycolysis and on glycolytic capacity.

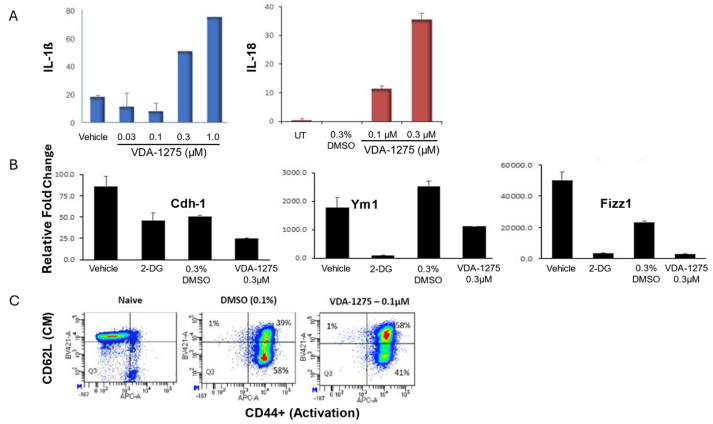


Figure 3: VDA-1275 shifts macrophages phenotype from M2 to M1 and elevates central memory CD8$^+$ T-cells population. (A): Secretion of the cytokines 1L-1ß and IL-18 from recombinant Macrophage Colony Stimulating Factor (M-CSF) grown BMDMs cells, primed with LPS and peptidoglycan in the presence of VDA-1275; (B): BMDM cells expression of M2-phenotype genes Fizz1, Ym1 and Cdh-1, studied with real time PCR, in the presence of VDA-1275 and 2-DG; (C): Population of central-memory CD8 T-cells (CD44, CD62L biomarkers) from splenocytes isolated from a transgenic mouse (that carries a T-cell receptor transgene specific for the mouse homologue pmel-17) in the absence or presence of VDA-1275 (0.1µM); FACS analysis.

VDA-1275 increases Central Memory CD8 T-cell population *ex-vivo*

To study the effect of VDA-1275 on the activity of CD8 T-cells that are major players in the immune response against tumor cells, we isolated splenocytes from a transgenic mouse strain that carries a T-cell receptor transgene specific for the mouse homologue pmel-17, that can be stimulated by a specific peptide. Mouse splenocytes were primed with the peptide for 24 hours in the absence or presence of VDA-1275 (0.1µM). Cells were analyzed on FACS for their activation state using CD44 and for the Central Memory biomarker CD62L. While VDA-1275 did not affect CD8 T-cell activation or survival, a clear increase (about 49%) in the central memory CD8 T-cells population was observed (Figure 3C). These results may indicate a stronger immune memory acquired as a result of VDA-1275 treatment, that may reduce reoccurrence of metastases after treatment completion.

VDA-1275 treatment significantly reduces tumor growth *in-vivo*

In order to study the effect of VDA-1275 on cancer inhibition *in-vivo*, we used the MC38 syngeneic mouse model. C57BL/6 immuno-competent mice were inoculated subcutaneously at the right flank with MC38 cells for tumor development. Five days after tumor inoculation, 20 mice were selected and assigned into 2 groups using stratified randomization with 10 mice in each group based upon their tumor volumes. The treatments were started from the day of randomization (defined as D0) and included the following: (1) Vehicle group (25%PEG400+75% Labrafac Lipophile WL 1349) PO, QD x 25 days (2) treatment group VDA-1275 300mpk PO, QD x 25 days. The tumor sizes were measured during the treatment period. Survival was monitored with tumor volume exceeding 2000 mm^3 as endpoint. The entire study was terminated on day 25. Syngeneic models include immunocompetent mice and are known to lead to divergent responses. Tumor Growth Inhibition (TGI) was measured for both groups at day 11, the last day on which all were animals still alive (Figure 4A). The overall TGI on day 11 for the treatment arm *vs.* vehicle was 47% (p=0.02). Observing the individual curves for the VDA-1275 300mpk group suggests that the group could be divided into 'responders' (n=7; tumor size <400 mm3 on day 11) and 'non-responders' (n=3; tumor size>1000 mm^3 on day 11). The TGI for the 70% of the mice in the 'responders' sub-group was 80% Tumor Growth Inhibition (TGI) (p<0.0001). Treatment was well-tolerated without any adverse effect observed

in the MC-38 tumor-bearing mice. Time-to-endpoint Kaplan-Meier survival analyses shows that Vehicle group median survival was 15 days while treatment group median survival was 21 days (survival prolongation of 6 days), p=0.0147 (Figure 4B).

VDA-1275 demonstrate a synergistic effect in combination with other anti-cancer drugs in 3D human in-vitro model on BIOMIMESYS® hydroscaffold

To study and quantitate the effect of VDA-1275 in combination with existing first-line treatments for solid tumor on cancer cells, the human hepatocellular carcinoma cell line HepG2 was used in a 3D organoids model using BIOMIMESYS® matrix [20]. As shown in Figure 5A, VDA-1275 shows a dose-dependent decrease of proliferation rate, as well as of viability (as determined by viability rate or number of live cells/plate).

Next, two anti-cancer drugs were chosen to study their combination effect with VDA-1275. The first drug, Sorafenib, is a kinase inhibitor antineoplastic drug that blocks cell growth. The second is the chemotherapy drug Cisplatin. A preliminary experiment was performed to determine the IC50 of each drug using HEPG2 cells. The cells were incubated for 72 hours with increasing concentrations of the two anti-cancer drugs, followed by EdU staining for proliferation and DRAQ7 for viability tests. Viability Results show IC50 of 9.7μM for Sorafenib and no effect for Cisplatin, whereas IC50 for proliferation were about 2.4 μM and 15 μM for Sorafenib and Cisplatin, respectively. IC50 for number of live cells/plate was 0.6 μM for Cisplatin and 2.2 μM for Sorafenib.

In order to study the combined effect of VDA-1275 with the two anti-cancer drugs, we used Sorafenib at 3μM and Cisplatin at 5μM. Cell media with no drug was added as vehicle control. For each treatment, increasing concentrations of VDA-1275 (3-1000 nM) or vehicle were added. Cells were incubated for 3 days followed by viability and proliferation tests, as mentioned above.

Synergistic effect was defined using the Response additivity method. This reference model is also known as Linear Interaction Effect [18] and assumes that a positive interaction occurs when the combination of drug A and drug B elicits a greater effect than the sum of the individual drug's effects (EAB>EA+EB), termed potentiating synergistic effect. In addition, when the drug combination is greater than each of the individual drug's effects, but smaller than their sum, a partial additive effect is observed

An important aspect of combination therapy is the ability to reduce the drug doses of harmful drugs. Sorafenib was used at 3μM with VDA-1275 (1000 nM) to reach IC50 effect on viability rate, while when Sorafenib was tested alone a dose of 9.7μM was required for the same effect. Cisplatin was used at 5μM with VDA-1275 (300 nM) to reach 74.4% reduction of live cells number per plate, while even a dose of 100μM was not enough to reach such an effect when Cisplatin was used alone (Figure 5B).

Proliferation results demonstrated no potentiating synergistic effect. However, surprisingly, when the combination effect of Sorafenib or Cisplatin with VDA-1275 was tested on Percent of viability or number of live cells per plate, respectively, a clear potentiating synergistic effect was detected. Table 1 and Table 2 show the synergism effect of VDA-1275 with Sorafenib and with Cisplatin on tumor cell viability, and number of live cells, respectively.

In addition, partial additive effects were found for the combination of Sorafenib and VDA-1275 on number of live cells/well, and for the combination of Cisplatin and VDA-1275 on proliferation rate (Table 3 and Table 4 respectively).

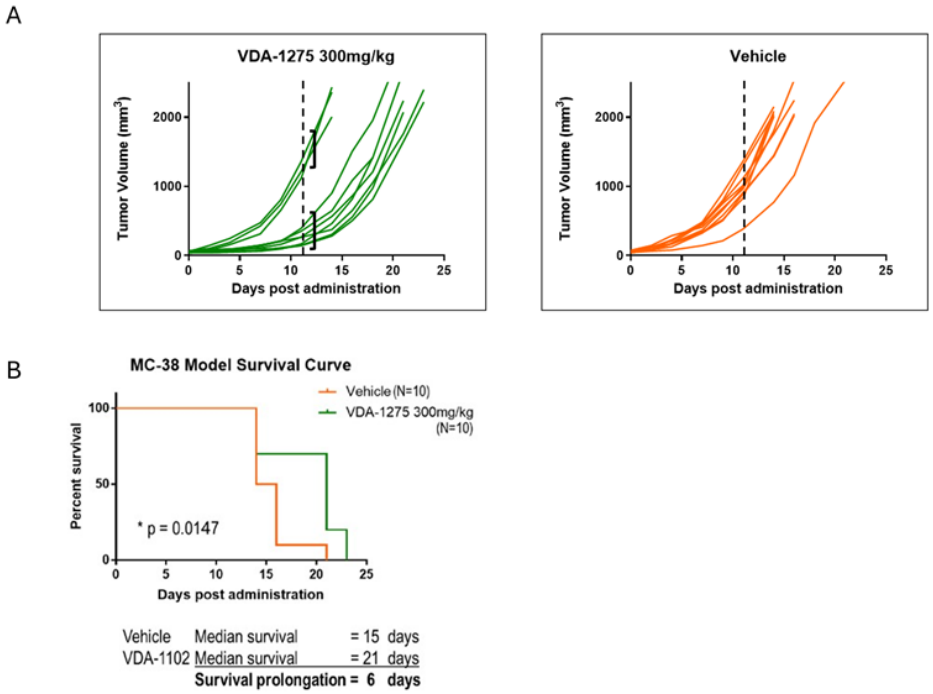


Figure 4: VDA-1275 reduces tumor growth and enhances survival *in-vivo*. (A): Tumor growth of C57BL/6 mice bearing MC-38 tumors treated with vehicle and VDA-1275, PO QD. When the mean tumor volume reached approximately 46 mm³, animals were randomized into treatment groups (n=10/group) and dosing was initiated on Day 0 of the study. Tumor size as measured by vernier calipers. Individual tumor growth curves are shown. Tumor Growth inhibition is measures on day 11; (B): Kaplan-Meier survival analyses comparing treatment groups. Mice were euthanized when tumors reached 2000 mm³.

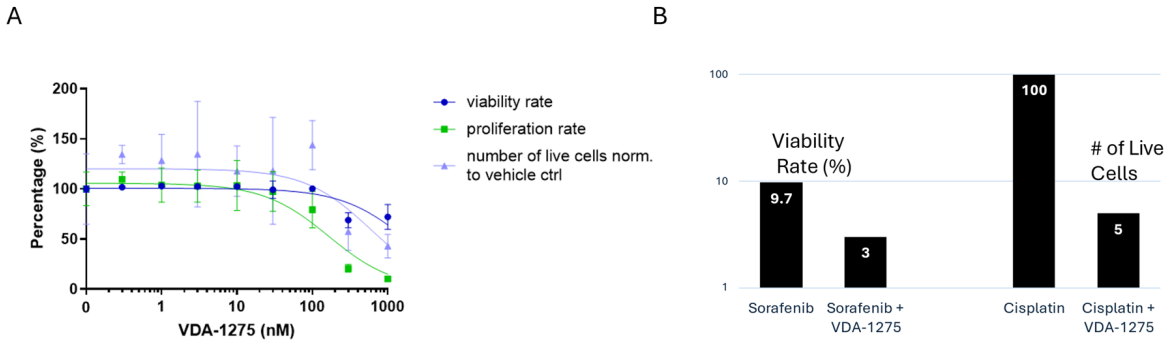


Figure 5: VDA-1275 decreases cancer cells viability and proliferation and has a synergistic effect with Cisplatin and Sorafenib. (A): Dose-dependent curves of VDA-1275 effect on proliferation rate and viability (viability rate & number of live cells/plate) using HepG2 cells in a 3D organoids model; (B): Doses of Sorafenib or Cisplatin without or with VDA-1275, required to reduce 50% viability rate or 74% number of live cells/plate, respectively.

Table 1: Potentiating synergistic effect of VDA-1275 and Sorafenib.

	Viability rate (%)	% inhibition
Vehicle control	100.00%	0.00%
VDA-1275 1000nM	72.10%	27.90%
Sorafenib 3μM	91.30%	8.70%
VDA-1275 1000nM+Sorafenib 3μM	46.80%	53.2%*
SUM VDA-1275 1000nM alone and Sorafenib 3μM alone		36.60%
Note: *Potentiating synergistic effect (ΣE>EA+EB)		

Table 2: Potentiating synergistic effect of VDA-1275 and Cisplatin.

	Number of live cells (norm to vehicle ctrl)	% inhibition
Vehicle control	100.00%	0.00%
VDA-1275 300nM	57.40%	42.60%
Cisplatin 5μM	96.50%	3.50%
VDA-1275 300nM+Cisplatin 5μM	25.60%	74.4%*
SUM VDA-1275 300nM alone and Cisplatin 5μM alone	-	46.10%
Note: *Potentiating synergistic effect (ΣE>EA+EB)		

Table 3: Partial additive synergistic effect of VDA-1275 and Sorafenib.

	Number of live cells (norm to vehicle ctrl)	% inhibition
Vehicle control	100.00%	0
VDA 1000nM	42.80%	57.20%
Sorafenib 10μM	26.30%	73.70%
VDA 1000nM+Sorafenib 10μM	16.10%	83.9%*
SUM VDA 1000nM alone and Sorafenib 10μM alone	-	131%
Note: *Partial additive synergy (ΣE<EA+EB)		

Table 4: Partial additive synergistic effect of VDA-1275 and Cisplatin.

	Proliferation rate	% inhibition
Vehicle control	100%	0
VDA 300nM	20.70%	79.30%
Cisplatin 5μM	80.50%	19.50%
VDA 300nM+Cisplatin 5μM	8.60%	91.4%*
SUM VDA 300nM alone and Cisplatin 5μM alone	-	99%
Note: *Partial additive synergy (ΣE<EA+EB)		

Our data suggests that VDA-1275 can be combined with Cisplatin or Sorafenib at lower concentrations and lead to a synergistic effect that results in a stronger antineoplastic outcome with less potential for adverse effects due to lower concentration of Cisplatin or Sorafenib as examples for chemotherapy and targeted therapy drugs.

VDA-1275 demonstrates a synergistic effect in combination with Cisplatin *in-vivo*, using a colon cancer syngeneic mouse model

To demonstrate *in-vivo* a synergistic effect of VDA-1275 with a standard of care anti-cancer drug, a syngeneic mouse model of colon cancer with an intact immune system was used. BALB/c-derived CT-26 colon carcinoma cells were transplanted in 8-10 weeks old BALB/c female mice. Six study groups (n=10) were used: A vehicle negative control (group 1), 0.5 mg/kg Cisplatin (group 2), 0.5 mg/kg Cisplatin and 400 mg/kg VDA-1275 (group 3), 1 mg/kg Cisplatin (group 4), 1 mg/kg Cisplatin and 400 mg/kg VDA-1275 (group 5), and 400 mg/kg VDA-1275 alone (group 6). Mice were treated with Cisplatin twice a week by the intraperitoneal rout of administration (IP), and with VDA-1275 daily using oral administration (PO). Doses and route of administration are listed in table 5 below.

VDA-1275 demonstrates a significant effect on tumor growth inhibition when combined with Cisplatin *in-vivo*.

The mice were randomized when tumors reach an average size of 50 mm^3, and treatments were started from the day of randomization (defined as D1). Tumor sizes were measured during the treatment period. Survival was monitored, with a tumor volume exceeding 1800 mm^3 used as the endpoint. Tumor Growth Inhibition (TGI) was measured. Treatment was well-tolerated without any observed adverse effects.

Figure 6A shows tumor size through treatment day 22 (first day of mice in the vehicle control group reached a tumor volume exceeding 1800mm^3). Since treatment with 0.5 mg/kg Cisplatin, with and without VDA-1275, demonstrated only a minor effect at this stage, these results are not shown in Figure 6A. The strongest growth inhibition versus vehicle control, although not statistically significant, was obtained with 1 mg/kg Cisplatin and VDA-1275 combined. VDA-1275 alone has a numerically greater tumor growth inhibition than 1 mg/kg Cisplatin alone, in this model. An additional comparison of tumor size was done on treatment day 38 (Figure 6B). For this analysis, the Last Observation Carried Forward (LOCF) method was applied to handle missing data (i.e., for animals whose tumor volume exceeded 1800 mm^3 before D38). As shown in Figure 6B, a significant inhibition of tumor growth relative to vehicle-treated animals was found in animals treated with 0.5 mg/kg Cisplatin together with 400 mg/kg VDA-1275, 1 mg/kg Cisplatin alone, and 1 mg/kg Cisplatin together with 400 mg/kg VDA-1275, which led to the highest growth inhibition.

VDA-1275 in combination with Cisplatin enhances median survival *in-vivo*

The combination treatment of 400 mg/kg VDA-1275 and 1 mg/kg Cisplatin demonstrated a highly statistically significant median survival relative to the vehicle control group. Median survival of the control group was 26 days while median survival for the group treated by 1 mg/kg Cisplatin together with 400 mg/kg VDA-1275 was 45 days, over 70% extension of median survival (p=0.0041). When the animals were treated with 400 mg/kg VDA-1275 alone or with 1 mg/kg Cisplatin alone, there was a numerical, albeit not statistically significant, extension of the survival period relative to the vehicle control group, with median survival of 36 days (p=0.0762) and 31 days (p=0.1021), respectively (Figure 7).

Comparing the effect of VDA-1275 and Cisplatin platin as standalone drugs to their combination *in-vivo*

In addition to evaluating the effectiveness of the combined 1 mg/kg Cisplatin and 400 mg/kg VDA-1275 treatment on Tumor Growth Inhibition (TGI) and on survival, we compared the sum of the individual TGI effects of each drug as monotherapies to the TGI effect of the combination treatment, at the same doses. The TGI effects was measured at two timepoints: Day 22 and Day 38 (see details above). As shown in Table 6, the sum of the individual drug effects, used as monotherapies (right column) was lower than the effect of the combination treatment (marked in bold letters), suggesting a possible synergy between the two.

Table 5: Study groups for Cisplatin and VDA-1275 *in vivo* CT-26 colon cancer model.

Group	Treatment	Dose (mg/kg)	Route	Schedule
1	Vehicle	0	p.o.	QD
	Vehicle	0	i.p.	Q3.5D
2	Vehicle	0	p.o.	QD
	Cisplatin	0.5	i.p.	Q3.5D
3	VDA-1275	400	p.o.	QD
	Cisplatin	0.5	i.p.	Q3.5D
4	Vehicle	0	p.o.	QD
	Cisplatin	1	i.p.	Q3.5D
5	VDA-1275	400	p.o.	QD
	Cisplatin	1	i.p.	Q3.5D
6	VDA-1275	400	p.o.	QD
	vehicle	0	i.p.	Q3.5D

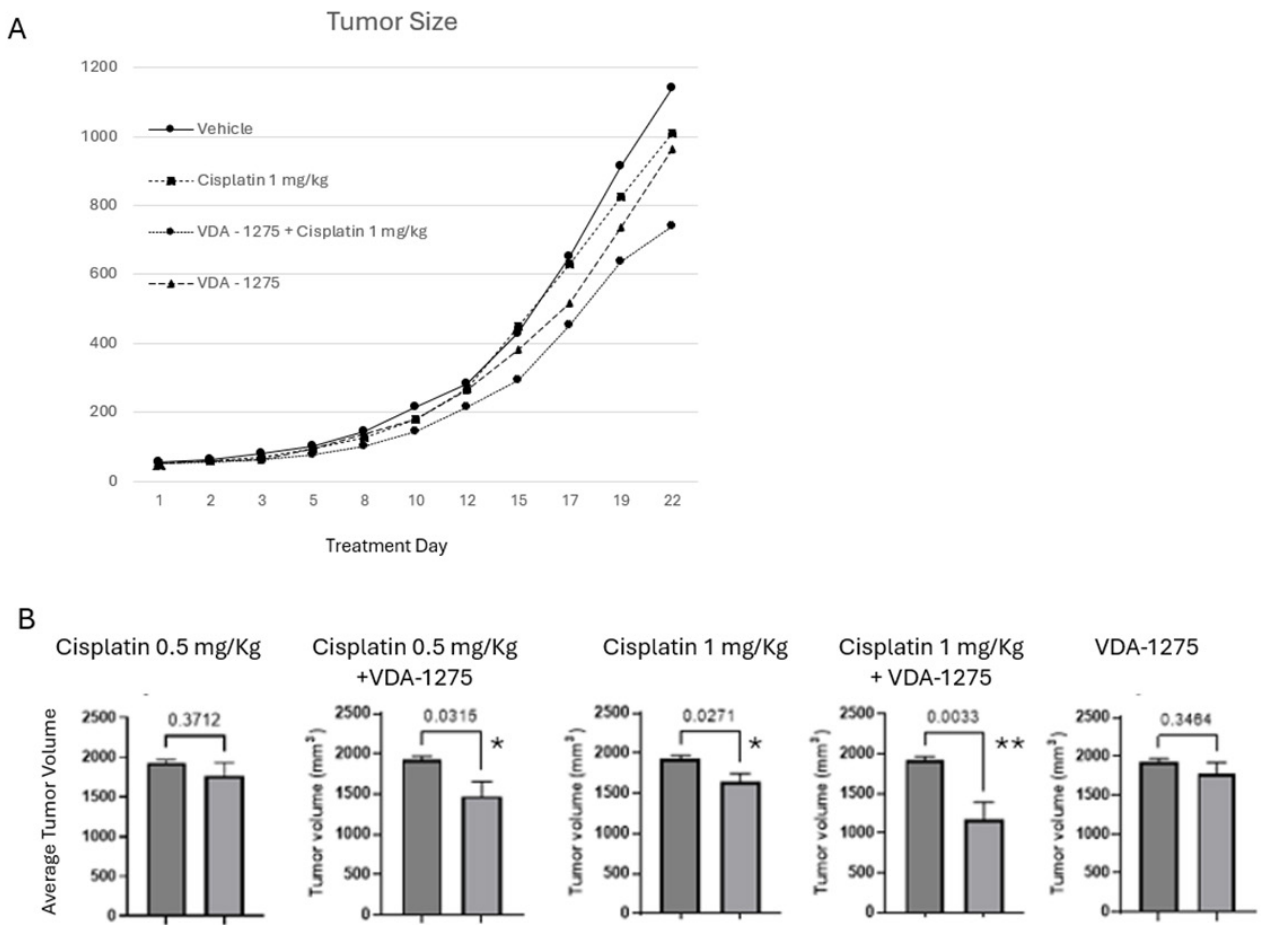


Figure 6: Tumor Size and Tumor Growth Inhibition (TGI%). (A): Tumor growth inhibition of BALB/c mice bearing CT-26 tumors (d22). When the mean tumor volume reached approximately 50 mm^3, animals were randomized into treatment groups, as described above (n=10/group) and dosing was initiated on Day 1 of the study. Tumor size as measured by vernier calipers; (B): Tumor growth inhibition (d38) using the last observation carried forward method. Note: * =p<0.05, **p<0.005.

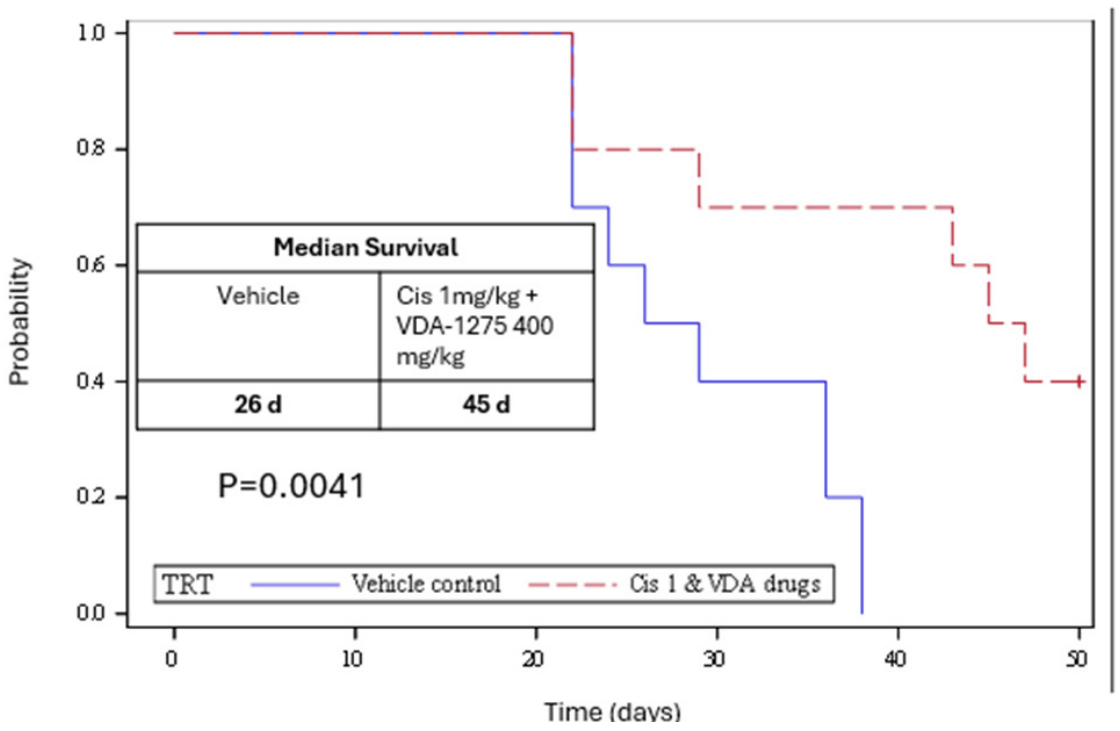


Figure 7: Kaplan-Meier and median survival analysis. Kaplan-Meier survival analyses comparing vehicle control group to 1 mg/kg Cisplatin IP and 400 mg/kg VDA-1275 PO combination treatment group. Mice were euthanized when tumors reached 1800 mm^3. Note: p<0.005.

Table 6: Combination of Cisplatin and VDA-1275 lead to a synergistic effect. To demonstrate a synergistic effect of Cisplatin (1 mg/Kg) and VDA-1275 (400 mg/Kg), the sum of the effect of each of the two drugs, as stand-alone, was calculated at each time point (mean TGI (%) right column) and compared to the combination treatment effect (mean TGI (%) Bold letters), at the same timepoint.

Treatment	Mean tumor size control (mm3)	Mean tumor size treatment (mm3)	Mean TGI treatment *vs.* control (%)	Sum of TGI for Cisplatin and VDA-1275 alone (%)
		Day 22		
1 mg/kg Cisplatin	912.4	824.6	9.6	-
VDA-1275	912.4	735.5	19.4	-
1 mg/kg Cis & VDA-1275	912.4	637.2	**30.2**	29
		Day 38		
1 mg/kg Cisplatin	1925	1640	14.8	-
VDA-1275	1925	1773	7.9	-
1 mg/kg Cis & VDA-1275	1925	1177	**38.9**	22.7

DISCUSSION

The Warburg effect, which characterizes one of the initial and essential metabolic effects in the transformation to malignancy, is highly associated with poor outcome [21]. The transfer from oxidative phosphorylation to glycolysis metabolism in cancer cells is mediated by HK2, a major player in both the Warburg effect and cancer cell immortalization. HK2 is known to be highly expressed in most cancer cells and its effect involves interaction with the mitochondrial outer membrane VDAC1 channel. Although it was discovered about 100 years ago, no treatment was developed that can block this process, without affecting surrounding normal tissue.

VDA-1275 direct effect on cancer cells

Our data demonstrates that upon treatment of cancer cells with VDA-1275, there is a strong decrease in glycolysis and cell proliferation (Figure 2). An additional direct effect of VDA-1275 on cancerous cells is reintroduction of apoptosis. Highly proliferating cancer cells are under stress conditions that may lead to apoptosis through the release of pro-apoptotic protein from mitochondria to cytoplasm. The interaction of HK2, which is a large protein (~100KDa), with the VDAC1 channel blocks the release of pro-apoptotic molecules like cytochrome C and induces apoptosis resistance. Upon addition of VDA-1275 to cancer cells, there is an immediate elevation of apoptosis (Figure 1B). This reintroduction of sensitivity to apoptosis, may lead to improvement of the immune system effect, as well as anti-malignant drugs effects on cancer cells in the TME of the solid tumor.

VDA-1275 effects on TME and immune cells

The direct effect of VDA-1275 on cancer cells also changes the tumor microenvironment from a pro-tumor to an anti-tumor/pro-immunologic TME. The effect of VDA-1275 is multi-faceted and includes direct anti-cancer activity as well as effects on the TME. VDA-1275 interferes with the interaction of HK2 with VDAC1, which in turn blocks hyper glycolysis and cancer cell high rate of proliferation. Blocking hyper glycolysis inhibits lactate fermentation which stops the exploitation of intermediate carbon chains for biomass generation and other intermediate products required for cancer cell growth [1]. In addition, it lowers the secretion of lactate and the low pH in the TME. Until recently, lactate was regarded only as a metabolic waste product of the glycolysis process. However, recent studies show that lactate sustains not only the survival of cancer cells but also immune cells. However, it also inhibits the function of Tumor-Associated Macrophages (TAMs), thereby facilitating the immune evasion of malignant tumor cells [19]. TAMs are generally divided into the pro-inflammatory M1 phenotype and the pro-tumor M2 phenotype. In the presence of elevated levels of lactate TAMs tend to adopt an immunosuppressive phenotype and collaborate with tumor cells to promote angiogenesis [19]. In addition, lactic acid suppressed the proliferation and cytokine production of human cytotoxic T lymphocytes up to 95% and led to a 50% decrease in cytotoxic activity [22]. VDA-1275 effect on the TME allows better efficiency of the immune system and elevates the effect of anti-cancer drugs that induces apoptosis or aimed to enhance the immune system activity like immune checkpoint inhibitors.

In addition to the direct effect of VDA-1275 on cancer cells and the metabolic changes of the TME, we have shown that this compound changes the phenotypic activity of immune cells directly, even when cancer cells are not involved. Our data demonstrated that the addition of VDA-1275 to macrophages changes their phenotype from the tumor-supportive M2 type to the anti-tumor M1 type (Figure 3B). As for the role of glycolysis in T cells, it is known that after initial antigenic stimulation, T cells switch their metabolism to glycolysis to support the increase in cell size, proliferation, and effector functions [23]. When we added VDA-1275 to CD8 T-cells derived from a transgenic mouse strain that can be stimulated by a specific peptide, we found a significant increase in the subpopulation of central memory CD8 T-cells (Figure 3C). This elevation of immune memory for a specific antigen indicates a positive effect of VDA-1275 treatment that may prevent or decrease recurrence of metastases after treatment completion. A hint of this effect might have been observed with our topical VDA-1102 compound that shares the same mechanism of action of VDA-1275 and was shown to have similar effect on activated CD8 T-cells (not published). The results of our phase 2 clinical study of actinic keratosis showed no lesion grade progression in the treatment group, while the placebo group had two patients with lesion progression.

Combination therapy with VDA-1275

In this study the human hepatocellular carcinoma cell line HepG2 was used in a 3D organoid model, mimicking actual tumors, to

test whether a chemotherapy drug (Cisplatin), and a targeted anti-cancer drug (Sorafenib) can induce a strong anti-proliferative and killing effects when used at their EC50, or even lower concentrations, in combination with VDA-1275. Combination therapy for the treatment of solid tumors is not a new concept. This approach potentially reduces drug resistance, while simultaneously providing therapeutic anti-cancer benefits, such as reducing tumor growth and metastatic potential, arresting mitotically active cells, reducing cancer stem cell populations, and inducing apoptosis [24]. Combination therapy should be used especially in advanced stages of the disease. For example, a meta-analysis that investigated the efficacy and safety of Immune-Checkpoint Inhibitors (ICIs) plus chemotherapy in patients with extensive-stage small cell lung cancer showed that ICIs plus chemotherapy was associated with significantly improved overall survival [25]. Several studies demonstrated specifically the advantage in combination of immunotherapy with drugs that changes the TME to support the anti-tumor effect [5-6].

To study combination of these drugs with VDA-1275, we first tested the dose response and EC50 for VDA-1275, Cisplatin and Sorafenib as single treatments. This was followed by combination therapy of VDA-1275 with Cisplatin or Sorafenib at their EC50 and lower concentrations. Synergistic effect was defined according to the response additivity method. A strong combination effect on cell proliferation was found when Cisplatin was combined with VDA-1275. The effect on proliferation did not lead to potentiating synergistic effect but only to a partial additive effect, where the combination of Cisplatin and VDA-1275 was greater than each of the individual drug's effects, but smaller than their sum effect. However, a clear potentiating synergistic effect was detected, when the combination effect of Sorafenib or Cisplatin with VDA-1275 was tested on percent of viability or number of live cells per plate, respectively.

To further demonstrate a synergistic effect of VDA-1275 with an anti-malignant therapy *in-vivo*, we used a CT-26 colon cancer syngeneic mice model with BALB/c mice. The combination treatment of VDA-1275 at 400 mg/Kg and Cisplatin at 1 mg/Kg show a significant improvement of tumor growth inhibition (Figure 6B), as well as median survival improvement relative to vehicle control group (45 days *vs.* 26 days, respectively). In addition, the combination therapy demonstrated a stronger effect on tumor growth inhibition than the sum of the two drugs effects when used as single treatment, at the same doses.

This mice model combination treatment potentially supports life elongation with less adverse effect symptoms due to the low level of chemotherapy treatment with only 1 mg/Kg Cisplatin. The same concentration of Cisplatin as a single therapy shows median survival of only 31 days. These results indicated that VDA-1275 with its strong safety profile, may be a promising combination therapeutic candidate, that can be synergized with current first-line treatments and leads to a stronger anti-cancer effect with less potential for adverse effect due to reduced concentration of the combined anti-cancer drugs, such as chemotherapy, targeted therapy, or immunotherapy. Additional studies of VDA-1275 combination treatments with other first-line malignancies treatments may allow further effective treatments with a better safety profile to future patients.

CONCLUSION

A key issue in treating solid tumors is their ability to evade the immune response and resist conventional therapies like chemotherapy, targeted, and immunotherapies. Tumor cells can manipulate TME to create an immunosuppressive environment. New treatment approaches are aimed to enhance immune responses against tumors such as immune checkpoint inhibitors, or to disrupt the supportive network within the TME such as targeting angiogenesis or inhibiting tumor supportive signaling. VDA-1275 is a promising novel small molecule drug that changes the metabolism of cancer cells by an allosteric effect that does not change activity but only the positioning of HK2 by blocking the binding site of HK2 to its harmful anchor-the VDAC1 mitochondrial channel. This new mode of action allows the return of HK2 molecules to their original cytosolic location and affects cancer cells directly, by blocking their proliferation and induction of apoptosis, without affecting normal cells and without harming the highly conserved active site in all Hexokinases. We coined this mode of action the "Toposteric effect". In addition to the direct effect on cancerous cells, VDA-1275 changes the TME to a pro-immunogenic environment and has a similar effect directly on CD8 positive T-cells and macrophages. VDA-1275 may be used as a standalone drug, or in combination therapy that will allow more effective and safe treatment of patients with solid tumors.

ETHICS APPROVAL

The animal study was performed by ChemExplorer Company, Shanghai ChemPartner Co., with their place of business at 998 Halei Road, Building Number 5, Shanghai 201203, China.

Observations: The study will be performed following to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of ChemPartner in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). After tumor inoculation, the animals will be checked daily for morbidity and mortality. At the time of routine monitoring, the animals will be checked for any effects of tumor growth and treatment on normal behavior such as mobility, body weight gain/loss (body weights will be measured twice weekly or every other day), eye/hair matting and any other abnormal effect. Death and observed clinical signs will be recorded. Any serious adverse reactions or death will be reported to Vidac immediately."

Data collection and updates: Tumors will be measured three times per week during the dosing period. The raw data of tumor size and body weight will be updated to Vidac at least twice a week.

Termination: Animals showing obvious signs of severe distress and/or pain should be humanely sacrificed. In case of following situation, the animals will be euthanized following discussion with Vidac.

- Animals have lost significant body mass (emaciated). Obvious body weight loss >20%
- Animals cannot get to adequate food or water.
- Tumor volume>2000 mm^3

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

COMPETING INTEREST

MH, YS are working at Vidac Pharma Ltd. and have a financial competing interest. OMB has financial interests in Vidac Pharma Ltd. All other authors have no competing interest.

FUNDING

Not applicable

AUTHOR'S CONTRIBUTIONS AND APPROVAL OF MANUSCRIPT

MH made substantial contributions in creating the concept and the descriptive word for Toposteric effect and to the conception and revision of the data. YS has drafted the article and managed the work that demonstrated the synergistic effect of VDA-1275 with other anti-cancer drugs. OMB and VB contributed to the conception and design of most of this work and have reviewed the article. RY, ED and HP contributed to the acquisition and analysis of the data. All authors read and approved the final manuscript.

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Not applicable

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